THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 9, Issue of March 4, pp. 6511–6516, 1994
Printed in U.S.A.
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Activation of the Skeletal Muscle Calcium Release Channel by a Cytoplasmic Loop of the Dihydropyridine Receptor*

(Received for publication, September 21, 1993, and in revised form, November 22, 1993)

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Expression studies with skeletal and cardiac muscle cDNAs have suggested that the putative cytoplasmic loop region of the dihydropyridine receptor (DHPR) α1 subunit between transmembrane repeats II and III (DCL) is a major determinant of the type of excitation-contraction coupling (skeletal or cardiac) in rescued dysgenic muscle cells (Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T., and Numa, S. (1990) *Nature* 346, 567–569). In this study, the possibility of a direct functional interaction with the sarcoplasmic reticulum ryanodine receptor/Ca^{2+} release channel has been tested by expressing the DCLs of the mammalian skeletal and cardiac muscle DHPR α1 subunit in *Escherichia coli*. The purified peptides activated the skeletal muscle ryanodine receptor/Ca^{2+} release channel in single channel and [^{3}H]ryanodine binding measurements, by increasing channel open probability and the affinity of [^{3}H]ryanodine binding, respectively. The two peptides did not activate the cardiac muscle Ca^{2+} release channel. Other proteins (polylysine, serum albumin) also increased [^{3}H]ryanodine binding and Ca^{2+} release channel activity, but their activation mechanisms were distinguishable from DCLs. These results show that the II-III cytoplasmic loop of the skeletal and cardiac DHPR α1 subunit functionally interacts with the skeletal, but not cardiac, muscle Ca^{2+} release channel. Furthermore, our studies suggest that in addition to the DHPR, the sarcoplasmic reticulum Ca^{2+} release channel may determine the type of E-C coupling that exists in muscle.

The mechanisms of excitation-contraction (E-C)[1] coupling in skeletal and cardiac muscle differ in their dependence on extracellular Ca^{2+}. In cardiac muscle, the entry of extracellular Ca^{2+} via a voltage-sensitive dihydropyridine receptor (DHPR)/Ca^{2+} channel is required to trigger Ca^{2+} release from the sarcoplasmic reticulum (SR) (1–3). In contrast, in skeletal muscle an action potential triggers the release of Ca^{2+} from SR in the absence of extracellular Ca^{2+} (4). As a consequence, skeletal muscle E-C coupling has been suggested to occur by a mechanical coupling mechanism involving protein-protein interactions between the voltage-sensing DHPR located in the tubular infoldings (T-tubule) of the surface membrane and the ryanodine receptor (RYR)/Ca^{2+} release channel located in the SR membrane (5).

The skeletal and cardiac RYRs are homologous channels comprised of four M_r ~ 565,000 polypeptides (6). Similarly, the two muscle DHPRs are related oligomeric complexes comprised of up to five different subunits (7). One of the major subunits (α1) binds Ca^{2+} channel antagonists, conducts Ca^{2+} ions, and displays significant sequence homology with the Na^+ channel. The main putative cytoplasmic regions of the DHPR α1 subunit include the amino- and carboxyl-terminal regions and the loop regions between transmembrane repeats I and II, and II and III. These cytoplasmic regions exhibit major differences in primary sequence between the skeletal (8) and cardiac (9) muscle DHPRs and accordingly represent regions of the DHPR that could confer tissue specificity to the functional interaction between the cardiac and skeletal DHPRs and RYRs.

Valuable insights into the sequences involved in the functional coupling of the DHPR and RYR have been obtained in expression studies with a dysgenic mouse model. Dysgenic skeletal muscle cultures are incapable of E-C coupling because they lack the α1 subunit of the DHPR (10). Injection of the skeletal α1 DHPR cDNA restored E-C coupling in the absence of extracellular Ca^{2+}, suggesting restoration of skeletal muscle-type E-C coupling (11). In contrast, cardiac-type E-C coupling was observed following the injection of cardiac α1 DHPR cDNA since its restoration required extracellular Ca^{2+} (12). Studies with cardiac and skeletal muscle chimeric cDNAs suggested that the II-III loop region of the DHPR α1 subunit is a major determinant for the type of E-C coupling (skeletal or cardiac) observed in dysgenic muscle cells (13), implying that this loop may functionally interact with the RYR. The interaction of the II-III loop region with the cardiac RYR and the role of the RYR isoforms in determining cardiac- or skeletal-type E-C coupling have not been described.

Here we report that the putative cytoplasmic loops between transmembrane repeats II and III of the skeletal and cardiac muscle α1 DHPR expressed in *E. coli* activate the skeletal but not the cardiac muscle Ca^{2+} release channel. Our studies suggest that, in addition to the DHPR, the SR Ca^{2+} release channel may determine the type of E-C coupling that exists in muscle.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Ryanodine (54.7 Ci/mmol) was obtained from DuPont NEN. Unlabeled ryanodine was from AgriSystems International (Wind Gap, PA). Plasmid pET11d, *E. coli* strain BL21(DE3) and T7·Tag monoclonal antibody were from Novagen (Madison, WI). DEAE-Sephacel, hydroxyapatite, SDS-PAGE molecular weight markers, bovine serum albumin (BSA), insulin, cytochrome *c*, polylysine (M_r 26,300), and polyglutamate (M_r 13,300) were purchased from Sigma, and phospholipids from Avanti (Birmingham, AL). All other chemicals were of analytical grade.

Expression and Purification of SDCL and CDCL—cDNAs encoding SDCL (Glu^{666} to Leu^{791}) (8) or CDCL (Asp^{788} to Gln^{922}) (9) were ampli-

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[1] The abbreviations used are: E-C, excitation-contraction; SR, sarcoplasmic reticulum; DHPR dihydropyridine receptor; RYR, ryanodine receptor; DCL, the putative cytoplasmic loop between transmembrane repeat II and III of DHPR α1 subunit; SDCL, skeletal muscle DCL; CDCL, cardiac muscle DCL; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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fied by reverse transcription PCR from rabbit skeletal and heart muscle mRNA, respectively. The nucleotide sequences of cloned PCR products were determined and showed agreement with those published. The PCR products were cloned into the *Bam*HI site of pET11d expression vector with in-frame insertion. The recombinant plasmids pET11d-sdcl or pET11d-cdcl were expressed in *E. coli* strain BL21(DE3) as described elsewhere (14). Cells were washed twice with 20 mM Na-Hepes, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and fragmented by sonication for 3 × 20 s using a 6-mm probe. After removal of particulate material by centrifugation at 60,000 rpm for 15 min in a Beckman TL100.3 rotor, the supernatant fraction was loaded onto a 5-ml DEAE-Sephacel column equilibrated with 20 mM Na-Hepes, pH 7.4, at 4 °C. The column was washed with 50 ml of 20 mM Na-Hepes, pH 7.4, and proteins were eluted with a 0–200 mM NaCl gradient. SDCL and CDCL were eluted at 100 and 180 mM NaCl, respectively. Peptide fractions were dialyzed against 1 mM potassium phosphate, pH 7.0, at 4 °C, loaded onto 2-ml hydroxyapatite column equilibrated with 1 mM potassium phosphate, pH 7.0, and eluted with a 1–300 mM potassium phosphate gradient. SDCL and CDCL were eluted at 80 and 60 mM potassium phosphate, respectively, dialyzed against 20 mM Na-Hepes, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin, concentrated with a Centricon 10 concentrator (Amicon, Beverly, MA), frozen in liquid nitrogen, and stored at −80 °C.

Isolation of SR Vesicles—Heavy SR vesicles were prepared from rabbit skeletal muscle and canine heart muscle in the presence of protease inhibitors as previously described (15, 16).

Purification and Reconstitution of 30 S RYR—The CHAPS-solubilized 30 S RYR complex was isolated by rate density gradient centrifugation in the presence of phospholipid and protease inhibitors (17). The purified RYR was reconstituted into liposomes by removal of CHAPS by dialysis for 40 h at 4 °C against 10 mM Na-Hepes, pH 7.4, buffer containing 0.5 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. The reconstituted RYR was sedimentated by centrifugation (200,000 × *g* for 2 h), resuspended in 20 mM Na-Hepes, pH 7.0, 0.25 M NaCl, 100 μM EGTA, 200 μM $CaCl_2$, rapidly frozen, and stored at −80 °C. Before use, samples were thawed, once more frozen and thawed, and sonicated for 3 × 20 s in a water bath sonicator to generate proteoliposomes with an average diameter of 1,500 Å.[2]

Single Channel Recordings—Single channel measurements were performed following the incorporation of the purified, CHAPS-solubilized skeletal muscle 30 S RYR/Ca^{2+} release channel complex into Mueller-Rudin type planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio of 5:3:2, respectively (50 mg/ml phospholipid in *n*-decane). A symmetrical solution of 20 mM K-Pipes, pH 7.0, 0.25 M KCl, 50 μM free Ca^{2+} was used to incorporate single channels. Before addition of DCLs, channel open probability (P_o) was lowered to <0.1 by decreasing the free Ca^{2+} concentration in the *cis* chamber by addition of EGTA. Recordings were made with an Axopatch-1D amplifier and filter setting at 5 kHz and stored on VCR tape. Unless otherwise specified, replayed data were digitized at the sampling rate of 5 kHz and filtered at 1.5 kHz. Channel open probabilities and lifetimes of open and closed events were determined by 50% threshold analysis as previously described (18).

[^{3}H]Ryanodine Binding—Unless otherwise indicated, SR vesicles or proteoliposomes containing the purified RYR were incubated at 12 °C for 20 h in 100 μl of reaction mixture containing 20 mM Na-Hepes, pH 7.4, 0.1 M NaCl, 100 μM EGTA, 125 μM $CaCl_2$, 5 mM AMP, 0.5 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 5 nM [^{3}H]ryanodine. Bound and free [^{3}H]ryanodine were determined as described elsewhere (19). Nonspecific binding was determined in the presence of a 1,000-fold excess of unlabeled ryanodine.

RESULTS

Expression and Purification of DCLs—The cytoplasmic loops between transmembrane repeats II and III of the DHPR α1 subunit (DCL) from rabbit skeletal muscle (SDCL) and rabbit cardiac muscle (CDCL) (Fig. 1*A*) were expressed in *E. coli*, along with an amino-terminal (12 amino acid residues) Tag sequence. Both peptides were present in the soluble fraction of the cellular extracts and purified as described under "Experimental Procedures." SDS-polyacrylamide gel electrophoresis indicated a purity of greater than 90% for SDCL, while CDCL was about 98% pure (Fig. 1*B*). The two peptides were identified

[2] H.-B. Lee and G. Meissner, unpublished studies.

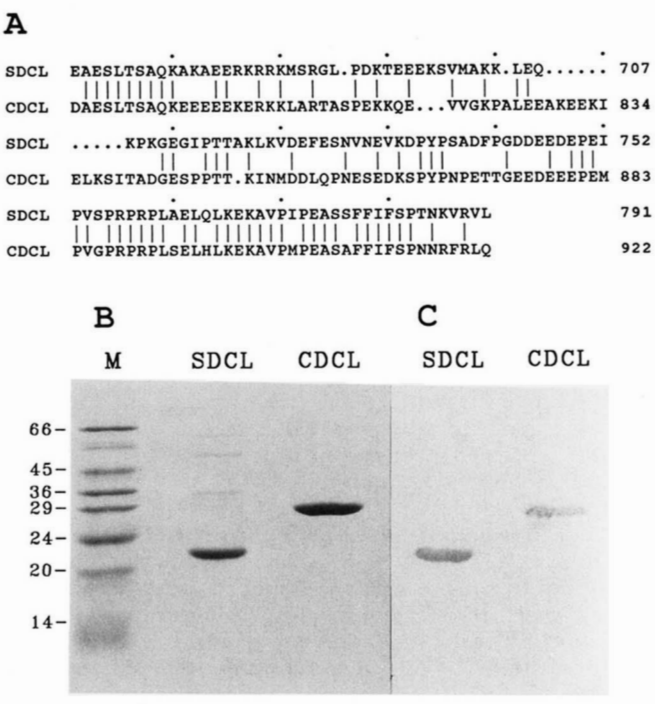

FIG. 1. **Analysis of purified SDCL and CDCL by SDS-PAGE and immunoblot.** *A*, comparison of deduced amino acid sequences of SDCL (Glu^{666} to Leu^{791}) (8) and CDCL (Asp^{788} to Gln^{922}) (9). Numbering of amino acid residues in α1 subunit of DHPRs is given on the *right*. *B* and *C*, purified proteins (5 μg) were run on a 12% polyacrylamide gel. Proteins were stained with Commassie Brilliant Blue R-250 (*B*), and following their transfer onto polyvinylidene difluoride membranes probed with T7·Tag monoclonal antibody (*C*). Molecular mass values (in kilodaltons) of standard proteins (*M*) are indicated on the *left*.

on Western blots using a monoclonal antibody directed against the NH_2-terminal Tag sequence present in the two DCLs (Fig. 1*C*).

Single Channel Recordings—The effects of a possible functional interaction of the purified SDCL and CDCL with the skeletal muscle RYR were first tested in single channel measurements. Single channels were recorded in symmetric 250 mM KCl medium, with K^+ as the current carrier. Since we found in preliminary experiments that the two peptides activated the RYR, channels were recorded with a suboptimally activating [Ca^{2+}] in the *cis* (SR cytoplasmic) chamber (Fig. 2*A*, *upper panels*). The two *middle panels* of Fig. 2*A* show that addition of 48 nM SDCL to the *cis* chamber resulted in an increase in Ca^{2+} release channel activity without an apparent change in single channel conductance. Three minutes after addition of the peptide, channel P_o increased from 0.04 to 0.71. A similar large activation was observed in four (out of four) experiments (Table I). Removal of SDCL by perfusion of the *cis* chamber decreased channel open probability to P_o = 0.003 (Fig. 2*A*, *bottom panels*), showing that the activation of the channel by SDCL was reversible. In other single channel recordings (not shown), addition of micromolar concentrations of ryanodine modified the K^+-conductance of the SDCL-activated channels in a manner highly characteristic for the skeletal Ca^{2+} release channel (19), thus confirming that ryanodine-sensitive channels were recorded in this study.

Fig. 2*B* depicts cumulative open and closed time histograms constructed from 7 s single channel recordings similar to those shown in Fig. 2*A*, *two left top panels*. In the absence of SDCL, the best fits were obtained using one and two exponential models for the open and closed time histograms, respectively. These results indicated the presence of at least one open and two closed channel states. The presence of additional short-lived

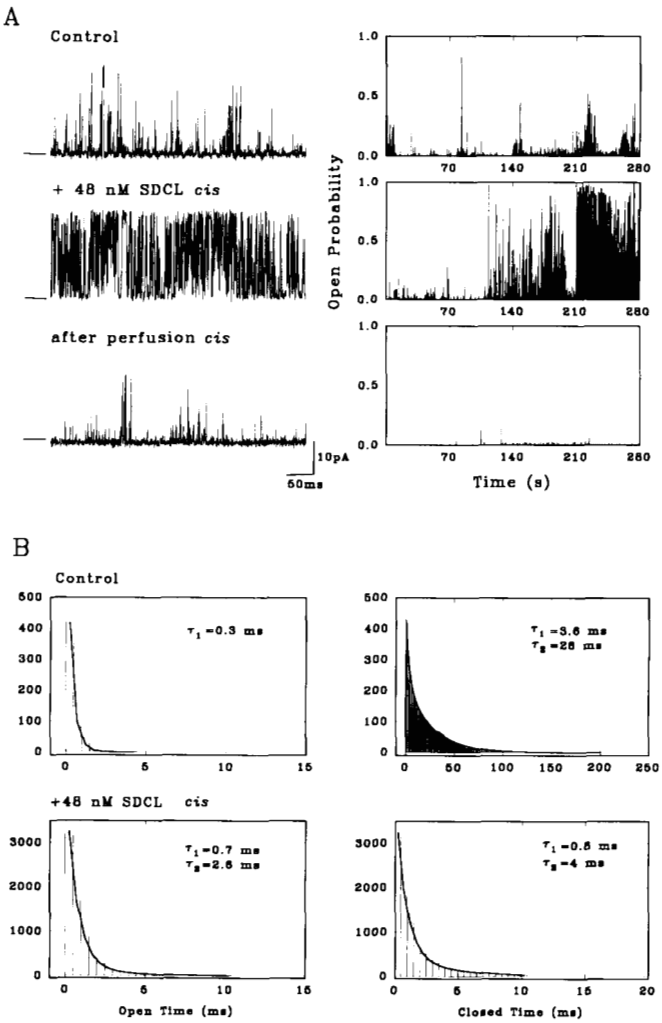

FIG. 2. **Effect of SDCL on single channel activity of the purified skeletal Ca^{2+} release channel.** *A*, single channel currents, shown as upward deflections, were recorded in symmetrical 0.25 M KCl media with 6.1 μM free Ca^{2+} *cis* and 50 μM free Ca^{2+} *trans*. *Left panels, top trace, Control*, P_o = 0.038; *middle trace*, 3 min after addition of 48 nM SDCL *cis*, P_o = 0.707; *bottom trace*, after perfusion of *cis* chamber with 0.25 M KCl buffer containing 6.1 μM free Ca^{2+}, P_o = 0.003. Holding potential = 30 mV. *Right panels* show values of P_o as fractional open times during successive sweeps of 250 ms. Zero times of the *middle* and *bottom panels* correspond to times of addition of SDCL and completion of perfusion, respectively. Sampling rate was 500 Hz and filter frequency was 100 Hz. *B*, cumulative open (*left panels*) and closed (*right panels*) time histograms of recordings in absence (*A*, *upper left panel*) and presence of 48 nM SDCL *cis* (*A*, *middle left panel*).

open and closed channel states was highly likely, however, since our bilayer frequency resolution limited detection of open and closed events to those with durations of 0.2 ms and longer. Addition of SDCL *cis* resulted in the appearance of a second resolvable open channel state and decreased the durations of the closed events (Table I).

It has been shown that the loop region between transmembrane spanning repeats II and III of DHPR α1 subunit is a major determinant of the type of E-C coupling that exists in muscle cells (13). Dysgenic mouse cells expressing α1 DHPR chimeras containing the skeletal muscle II-III loop exhibited skeletal-type E-C coupling, whereas cells expressing chimeras with the cardiac II-III loop showed cardiac-type E-C coupling. We found that *cis* CDCL was as effective as SDCL in activating the skeletal muscle Ca^{2+} release channel (Fig. 3*A*, *upper* and *middle panels*). A similar activation by CDCL was observed in five (out of five) experiments. The activation of the release channels by CDCL was reversible (Fig. 3*A*, *bottom panel*), as was observed for SDCL (Fig. 2).

In other experiments, 100 nM SDCL *cis* (n = 2) and 150 nM CDCL *cis* (n = 3) were without an effect on single channel activity of the canine cardiac Ca^{2+} release channel (not shown). Thus, single channel measurements suggested that the two DCLs directly interacted with the purified, reconstituted skeletal muscle Ca^{2+} release channel, but not the cardiac release channel.

The effects of BSA (Fig. 3*B*) and several other polypeptides (see "[^{3}H]Ryanodine Binding" below) were tested to assess the specificity of activation of the skeletal Ca^{2+} release channel by the two DCLs. BSA *cis* was less effective than the DCLs in activating single skeletal muscle release channels (Fig. 3*B*). Subsequent addition of SDCL further activated the channel, although to a lesser extent than in the absence of BSA. At concentrations ranging from 260–1700 nM, BSA activated the skeletal channel by a factor of 5.0 ± 3.8 (n = 5), as compared to 40 ± 10-fold activation by ~50 nM SDCL.

[^{3}H]Ryanodine Binding—The neutral plant alkaloid ryanodine has been shown to bind with nanomolar affinity to the mammalian Ca^{2+} release channels in a manner that correlates well with the functional state of the channels (20–22). Therefore, we also used [^{3}H]ryanodine binding to investigate the mechanism of activation of the Ca^{2+} release channel by SDCL and CDCL. The effects of the two DCLs on [^{3}H]ryanodine binding to the release channel were tested using conditions (see "Experimental Procedures") that resulted in ~10% occupation of the channel's high-affinity [^{3}H]ryanodine binding sites in the absence of added peptide. Addition of SDCL to heavy skeletal muscle SR vesicles increased [^{3}H]ryanodine binding in a dose-dependent manner, resulting in about 1.5-fold stimulation at 1 μM and a nearly 2-fold increase at 10 μM SDCL (Fig. 4). BSA was similarly effective, whereas CDCL was more effective than SDCL, increasing [^{3}H]ryanodine binding threefold at 10 μM. Low concentrations (1 μM) of polylysine (M_r 26,300) increased [^{3}H]ryanodine binding nearly 4-fold, whereas higher concentrations (10 μM) were less effective. A similar dose-dependent activation and inhibition of the skeletal muscle Ca^{2+} release channel by polylysine was observed in Ca^{2+} release measurements with heavy rabbit skeletal muscle SR vesicles (23).

Fig. 5 shows that SDCL and CDCL potentiated [^{3}H]ryanodine binding to the purified, reconstituted RYR. This result suggested that, in agreement with the single channel measurements, the effect of the two DCLs on the RYR is due to a direct interaction not mediated by other proteins. The specificity of the interaction of the two DCLs with the skeletal RYR was assessed by testing the effects of other randomly chosen polypeptides in potentiating [^{3}H]ryanodine binding and by using cardiac SR vesicles (Fig. 5). For comparative purposes, potentiation of [^{3}H]ryanodine binding to skeletal SR vesicles by BSA and polylysine (1 μM) already shown in Fig. 4, is included in Fig. 5. In contrast to these proteins, insulin, cytochrome *c* and polyglutamate (M_r 13,300) were without an effect. Fig. 5 further shows that the two DCLs did not significantly increase [^{3}H] ryanodine binding to cardiac SR vesicles. On the other hand, BSA and polylysine increased [^{3}H]ryanodine binding to cardiac SR vesicles. The extent of potentiation was less than that observed for skeletal SR vesicles, however.

SDCL and CDCL contained 12 additional amino acids in their NH_2-terminal ends. These additional residues were derived from the parent plasmid pET11d which encoded a 33-amino acid peptide. Although this peptide was expressed at high levels, as detected by immunodotblot analysis (not shown), it did not activate the RYR since extracts of the total soluble proteins from cultures of *E. coli* strain BL21(DE3) con-

TABLE I

Effect of SDCL on skeletal Ca^{2+} release channel open probability and lifetimes

Channel open probability (P_o) was calculated in files of 70 s, and events in files of 7 s. τ_o and τ_c represent the channel open and closed time constants, respectively. Data are shown as the mean ± S.D. of four experiments.

	P_o	τ_{o1}	τ_{o2}	τ_{c1}	τ_{c2}	Events
			ms			*n*
Control	0.021 ± 0.004	0.5 ± 0.3		6.7 ± 3.0	53.6 ± 23.8	259 ± 139
SDCL	0.717 ± 0.060 (after 3 min)	1.1 ± 0.3	5.7 ± 4.0	0.7 ± 0.1	6.8 ± 6.9	2073 ± 809

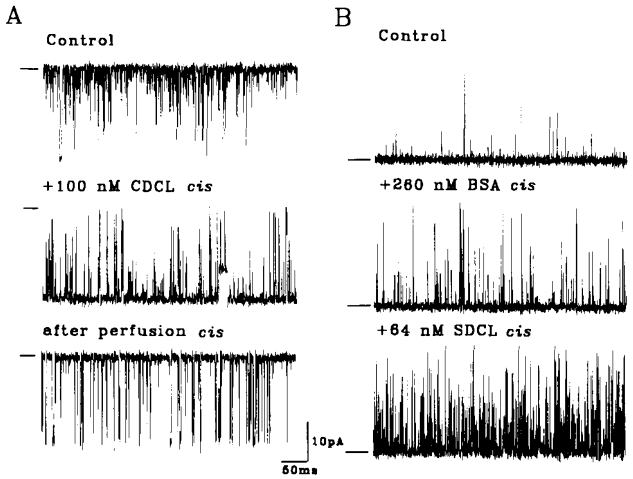

FIG. 3. **Effects of CDCL and BSA on single channel activity of the purified skeletal Ca^{2+} release channel.** *A*, single channel currents, shown as downward deflections, were recorded in symmetrical 0.25 M KCl media containing 50 μM free Ca^{2+}. Holding potential = −30 mV. *Top trace, control*, P_o = 0.057; *middle trace*, after addition of 100 nM CDCL *cis*, P_o = 0.920; *bottom trace*, after perfusion of *cis* chamber with 0.25 M KCl buffer containing 50 μM free Ca^{2+}, P_o = 0.099. *B*, single channel currents, shown as upward deflections, were recorded in symmetrical 0.25 M KCl media containing 9.7 μM free Ca^{2+} *cis* and 50 μM free Ca^{2+} *trans* (*top trace*, P_o = 0.005), following the successive addition of 260 nM BSA *cis* (*middle trace*, P_o = 0.019) and 64 nM SDCL *cis* (bottom trace, P_o = 0.099). Holding potential = 30 mV.

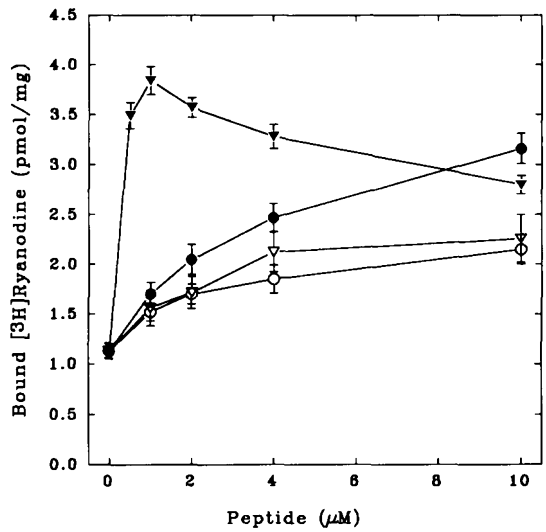

FIG. 4. **Effect of peptides on [^{3}H]ryanodine binding to skeletal SR vesicles.** SR vesicles (120 μg protein/ml) were incubated with 5 nM [^{3}H]ryanodine and indicated amounts of SDCL (*open circle*), CDCL (*closed circle*), BSA (*open triangle*), or polylysine (*closed triangle*) as described under "Experimental Procedures." Data (means ± S.D.) are the averages of three experiments carried out in duplicate. B_{max} value for [^{3}H]ryanodine binding to skeletal SR vesicles in the absence of peptides was 9.8 pmol/mg.

taining plasmid pET11d were without an effect on [^{3}H]ryanodine binding.

Scatchard analysis of [^{3}H]ryanodine binding data (Fig. 6) showed that SDCL and CDCL increased the binding affinity (K_D) without significantly affecting the B_{max} value of [^{3}H]ryanodine binding to skeletal SR vesicles (Table II). [^{3}H]Ryanodine binding was affected in a comparable manner by ClO_4^-, an activator of E-C coupling in skeletal muscle (24). In contrast, BSA significantly increased the B_{max} but not K_D value of [^{3}H]ryanodine binding (Fig. 6, Table II). The interaction of SDCL with the skeletal muscle Ca^{2+} release channel was also characterized by determining its activating effects in the presence of CDCL, BSA, and polylysine. The activating effects of 10 μM SDCL and 1 μM polylysine (Fig. 5) were additive, whereas no or only small further increases were measured in assay media containing 10 μM CDCL or 10 μM BSA (not shown). Taken together, the [^{3}H]ryanodine binding studies suggested that SDCL and CDCL likely activate the skeletal muscle Ca^{2+} release channel by a similar mechanism, but that there exist significant differences with regard to their action on the skeletal and cardiac muscle RYR isoforms.

DISCUSSION

Expression studies with dysgenic muscle cultures have shown that the putative cytoplasmic II-III loop of the skeletal and cardiac muscle DHPR α1 subunits plays a critical role in determining the mode of E-C coupling. In this study, the two DCLs were expressed and purified, and their functional interaction with the ryanodine receptor was examined *in vitro* using isolated membrane and receptor preparations. These studies led to two new observations that may have important implications for the E-C coupling mechanism in muscle. First, the data reported here provide the first evidence for a direct functional interaction of the cytoplasmic II-III loop of the DHPR α1 subunit with the SR Ca^{2+} release channel. Second, both SDCL and CDCL activated the skeletal, but not cardiac, release channel, indicating that in addition to the DHPR, the SR Ca^{2+} release channel may play an important role in determining the coupling mode in striated muscle.

Several other proteins have been suggested to associate with the DHPR and/or RYR, and thereby to possibly link or stabilize the interaction between the two receptor complexes. These include triadin, a SR membrane protein (25, 26), and glycolytic enzymes such as aldolase (27) and glyceraldehyde-3-phosphate dehydrogenase (28). The present study shows that SDCL activates the purified SR Ca^{2+} release channel, thus suggesting a direct interaction between the skeletal DHPR and RYR. To our knowledge, no direct functional or structural linkage between the DHPR and RYR has yet been demonstrated. One reason may be that the conditions that are required to maintain the two purified receptors in solution disfavor such an interaction.

Expression of cDNAs encoding chimaeras with the skeletal II-III cytoplasmic loop in dysgenic myocytes resulted in skeletal-type E-C coupling, whereas expression of cDNAs with the cardiac II-III loop elicited cardiac-type coupling (13). This suggested that of the two DCLs, SDCL should have been the more likely candidate of altering skeletal muscle Ca^{2+} release channel activity. An unexpected finding was therefore that both DCLs were able to activate the skeletal RYR. Indeed, of the two

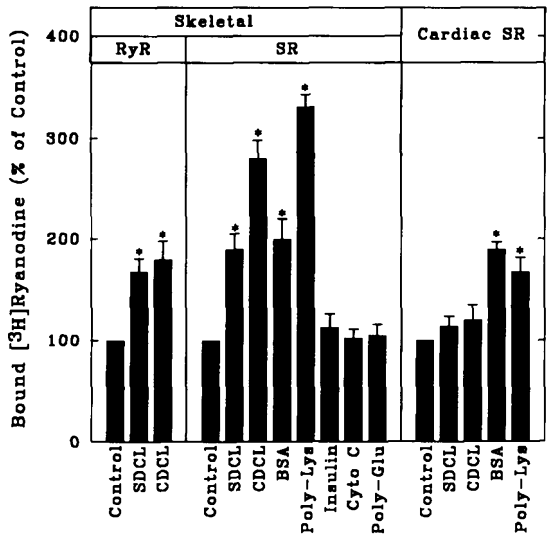

FIG. 5. **Comparison of effects of peptides on [^{3}H]ryanodine binding to reconstituted skeletal RYR, skeletal, and cardiac SR vesicles.** Purified, reconstituted skeletal RYR (8 μg/ml), skeletal SR vesicles (120 μg/ml), or cardiac SR vesicles (150 μg/ml) were incubated with [^{3}H]ryanodine (1 nM for purified RYR, 5 nM for vesicles) in the absence (*Control*), or presence of SDCL (10 μM), CDCL (10 μM), BSA (10 μM), polylysine (1 μM), insulin (160 μM), cytochrome *c* (80 μM), or polyglutamate (1 μM). Data (means ± S.D.) are the averages of three or more experiments carried out in duplicate. * Indicates a significant difference ($p < 0.05$) from control as determined by Student's paired *t* test.

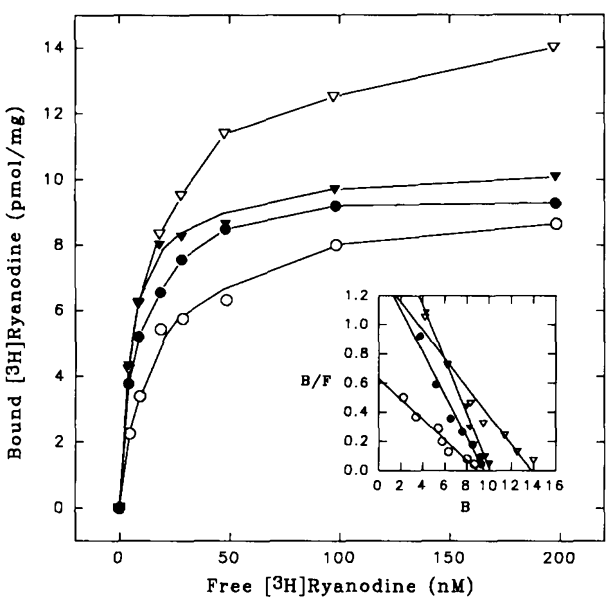

FIG. 6. **Effects of peptides on [^{3}H]ryanodine binding to skeletal SR vesicles.** Skeletal SR vesicles (120 μg/ml) were incubated with [^{3}H]ryanodine (5–200 nM) at 12 °C for 48 h in the absence of peptide (*open circle*), or presence of 10 μM SDCL (*closed circle*), 10 μM BSA (*open triangle*), or 10 μM CDCL (*closed triangle*). *Inset*, Scatchard plot of [^{3}H]ryanodine binding.

DCLs, the cardiac peptide appeared to be more effective in [^{3}H]ryanodine binding experiments. The amino acid sequences of SDCL and CDCL show 52% identity and 73% similarity (Fig. 1*A*). Accordingly, some of the sequences shared by the two DCLs are likely responsible for activating the RYR. Experiments of identifying these sequences are currently under way in our laboratory. Other portions of the DHPR α1 subunit such as the cytoplasmic loop between transmembrane regions I and II also have been reported to play a role, albeit a minor one, in determining the coupling mode in dysgenic muscle cells. It is

TABLE II

Effects of DCLs and BSA on B_{max} and K_D values of [^{3}H]ryanodine binding to skeletal SR vesicles

B_{max} and K_D values were determined by Scatchard analysis as described in Fig. 6. Results are given as the mean ± S.D. with number of experiments indicated.

Peptides	[^{3}H]Ryanodine binding		
	B_{max}	K_D	*n*
	pmol/mg protein	*nM*	
Control	9.2 ± 0.8	12.8 ± 0.8	4
SDCL (10 μM)	10.3 ± 1.3	7.1 ± 0.8[a]	3
CDCL (10 μM)	11.1 ± 1.4	7.0 ± 0.9[a]	3
BSA (10 μM)	14.0 ± 0.7[a]	10.1 ± 0.4	4

[a] Significant difference ($P < 0.05$) from control as determined by Student's paired *t* test.

possible that lack of these other interactions may result in a decreased specificity of DCL-RYR interaction. A partial loss of specificity would be also consistent with our observation that BSA activated the skeletal muscle RYR. Its action could be distinguished from that of the DCLs, however, by analyzing the mechanism of potentiation of [^{3}H]ryanodine binding by Scatchard analysis (Fig. 6). Whereas BSA increased the binding capacity (B_{max}) without significantly affecting the binding constant (K_D), the two DCLs increased the K_D value without affecting the B_{max} value, implying that the open-channel configuration induced by the DCLs (Figs. 2 and 3) exhibits an increased ryanodine binding affinity. Also, in contrast to the DCLs, BSA and polylysine were able to activate the cardiac RYR as well (Fig. 5). Furthermore, ineffectiveness of cytochrome *c*, insulin, and polyglutamate in altering skeletal RYR activity ruled out a ubiquitous activation of the skeletal muscle RYR by proteins and polypeptides.

Significantly higher concentrations of the DCLs were required to activate the RYR in [^{3}H]ryanodine binding experiments than in single channel measurements. Although the reasons for these differences are not well understood, they are consistent with other observations in our laboratory which have shown a higher sensitivity of the channel following its reconstitution into planar lipid bilayers of the Mueller-Rudin type. In bilayer measurements, activation of single channel activity by SDCL and CDCL was fully reversible. This excludes the possibility of a chemical modification reaction by a contaminating protease or some other protein modifying enzyme.

The RYR ion channel has been purified as a 30 S protein complex comprised of four polypeptides of ~5000 amino acid residues each. There is morphological evidence to suggest that, at least in some skeletal muscle in a subpopulation of RYRs, each of the four subunits may be linked to one DHPR (29). The high extent of channel activation in the single channel measurements suggests that the DCLs may interact with these sites, although this remains to be demonstrated. Further studies are required to answer several additional questions: Why do the DCLs activate the RYR? Does the action of the DCLs correspond to that of the DHPRs during an action potential, or how does the binding of the DCLs to the RYR alters the regulation by other endogenous effectors? The very large subunits of the RYR presumably allow a complex pattern of regulation since functional studies have shown that the channel may be regulated, in addition to the DHPR, by various endogenous effectors including Ca^{2+}, Mg^{2+}, ATP, and calmodulin (30).

The role of the RYR in determining the coupling mode in skeletal and cardiac muscle was not investigated by Tanabe *et al.* (11–13), because all their studies were done with cells expressing the skeletal RYR isoform. We found that the skeletal but not cardiac RYR isoform was activated by the DCLs. This result supports the mechanical coupling model in skeletal muscle, and also implies an important role for both the DHPR

and Ca^{2+} release channel in determining the coupling mode in striated muscle.

Acknowledgments—We thank Geoffrey Mann for preparing the reconstituted Ca^{2+} release channel vesicles and Anita Cohn for preparing the SR vesicles. We also thank Ron Grunwald and Dr. Hui Xiong for helpful discussions and reading the manuscript.

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